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Effect of Carob Pod Extract on Cellulolysis, Proteolysis, Deamination, and Protein Biosynthesis in an Artificial Rumen[1]

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ABSTRACT

TAGARI, H. (National and University Institute of Agriculture, Rehovot, Israel), Y. HENIS, MUSHA TAMIR, AND R. VOLCANI. Effect of carob pod extract on cellulolysis, proteolysis, deamination, and protein biosynthesis in an artificial rumen. Appl. Microbiol. **13**:437-442. 1965.—Carob pod extract and its tannin and sugar fractions were compared with gallotannic acid and sucrose for their effect on the cellulolytic, proteolytic, protein biosynthetic, and deaminative activities of rumen microorganisms. The inhibitory effects of carob pod extract upon the cellulolysis and deamination were correlated mainly with its sugar, rather than its tannin components. On the other hand, proteolytic activity and protein biosynthesis were more significantly affected by the tannin fraction. In contrast to the tannin fraction of carob pod extract, gallotannic acid inhibited cellulolytic activity. The harmful effect of a low concentration of tannins on protein biosynthesis could be prevented by the addition of carbohydrates to the reaction mixture. At high tannin concentration (40 μg/ml), however, the addition of carbohydrates did not prevent the inhibition.

Carob pods (the fruit of *Ceratonia siliqua*) have been used extensively, especially in the Mediterranean region, for feeding livestock. During the past decade, many reports have appeared concerning the detrimental effect of carob pods fed to lactating cows (Volcani and Roderig, 1961; Drori and Volcani, 1962), growing calves (Volcani and Drori, 1962; Volcani and Levi, 1962), and poultry (Alumot, Nachtomi, and Bornstein, 1964).

This detrimental effect of carobs has been attributed to their low caloric value in poultry nutrition (Alumot et al., 1964), low protein digestibility in lactating cows (Drori and Volcani, 1962; Oslage and Becker, 1958), and lowered digestibility of all ration components for calf nutrition (Drori and Volcani, 1963). In vitro experiments conducted in an artificial rumen have shown that carob pod meal and its various extracts inhibit cellulolysis and deamination (Volcani, Tagari, and Ascarelli, 1963). This phenomenon was also observed in vivo after prolonged feeding of carob meal (Y. Rabi, *personal communication*). Carob pods have a high tannin (ca. 1.0%) and sugar (ca. 40%) content. Natural tannins and tannin-containing substances have long been known to inhibit decomposition processes of organic matter (Basaraba, 1960; Oslage and Becker, 1958; Siu, 1951). Carob pod extract and its tannin, as well as gallotannic acid at very low concentrations, have been shown to inhibit saprophytic cellulolytic bacteria and modify their colonies, especially *Cellvibrio* (Henis, Tagari, and Volcani, 1964). A high soluble sugar content is also known to affect microbial activity (Krogh, 1954; Siu, 1951; Berman and Rettger, 1918).

Nitrogen balance trials showed an increase in the amount of nitrogen excreted in cattle (Drori and Volcani, 1963) fed carobs. It is not clear from previous studies whether the lower ammonia concentration in the artificial rumen, as compared with control treatment, was due to lower proteolysis or to an increased rate of ammonia uptake for protein synthesis by rumen microorganisms.

The purpose of this work was to elucidate further the effect of the tannin and sugar fractions of carob extract, as compared with gallotannic acid and sucrose, on the cellulolytic and proteolytic activities of rumen microorganisms in vitro.

MATERIALS AND METHODS

Rumen fluid of sheep (Awassi breed) was used as a source of rumen microorganisms. The sheep were fed the following diet (kilograms per day):

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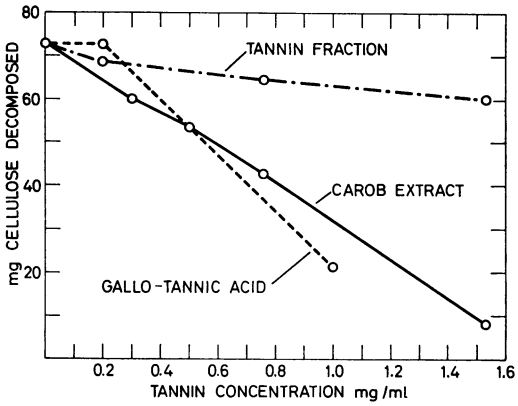

FIG. 1. *Effect of increasing concentrations of carob extract, its tannin fraction, and gallotannic acid on the in vitro cellulolytic activity of rumen fluid.*

alfalfa hay, 0.5; concentrates, 0.5; and cotton seed hulls, 0.5. Rumen fluid was obtained by means of a stomach tube, as described and modified by Tagari et al. (1964). Carob pod extract was prepared by refluxing carob pod meal in water (1:2.5, w/v) for 2 hr. The water extract was then cleared from remaining particles by centrifugation.

Tannin and sugar fractions of the pod extract were prepared as described by Henis (Henis et al., 1964).

Quantitative determination of tannins was carried out by the method of Rosenblatt and Peluso (1941), with the use of Folin's reagent.

Sugars were determined by the copper reduction method of Fehling (Browne and Zerban, 1955).

Cellulolytic activity of the rumen fluid was determined by a modification of the method of Crampton and Maynard (1938) for residual cellulose.

A 100-mg amount of cellulose powder (Whatman, coarse grade) was weighed into screw-cap test tubes (16 by 18 mm), to which 18 ml of the reaction mixture were added. The reaction mixture was composed of 20% rumen fluid and a mineral mixture, prepared as described by Halliwell (1957). Anaerobic conditions were obtained by the addition of cysteine (0.06 mg/ml, final concentration), and by the constant bubbling of CO_2 through the reaction mixture while filling the test tubes by means of an Adam's aupette. Resazurin (0.0002%, w/v, final concentration) was also added as a redox indicator (Bryant and Burkey, 1953). Test tubes in which a red color appeared, showing inadequate anaerobic conditions, were discarded. After an incubation period of 48 hr at 37 C, the residual cellulose was determined by a modified Crampton's method; the undecomposed cellulose was precipitated by centrifugation, and the supernatant fluid was discarded. To the precipitate, 5 ml of glacial acetic acid and 0.5 ml of concentrated nitric acid were added, and refluxed for 20 min, after which 5 ml of ethyl alcohol were added to each tube and the mixture was centrifuged for 10 min. The precipitate was washed with 5 ml of hot benzene, centrifuged, and washed again with hot ethyl alcohol. The washed precipitate was dried at 100 C for 24 hr and weighed. The whole procedure was carried out in the original test tubes.

Proteolytic activity of the rumen fluid was tested as described by Blackburn and Hobson (1960); test tubes and amounts of the reaction mixture were as described above for cellulolysis. The reaction mixture was composed of 20% rumen fluid in 0.2 M phosphate buffer (*p*H 7.0). Anaerobic conditions were attained as described above.

During the incubation period, coagulation of the reaction mixture sometimes occurred. To obtain a uniform suspension of the insoluble component, the contents of the test tubes were passed through a tissue homogenizer prior to the determination of residual protein. The residual casein was determined by the Biuret method for protein determination, as applied by Blackburn and Hobson (1960).

Determination of ammonia-nitrogen was conducted by the Conway (1957) method, in which a 20% NaOH solution was used to release the ammonia from the reaction mixture.

Protein-nitrogen was precipitated by trichloroacetic acid as described below. Nitrogen in the precipitate was then determined by the Conway (1957) method, except that the dishes were aerated for 20 min and covered with a glass plate at a height of 5 cm.

Protein biosynthesis and formation of free ammonia were tested in 18-ml screw-cap test tubes containing a growth medium composed of 8.25 g of Vitamin Free Casamino Acids (Difco); 75 mg of cysteine, and 150 mg of tryptophan and 375 mg of methionine dissolved in 100 ml of the mineral solution of Blackburn and Hobson (1960). Anaerobic conditions were obtained by the constant bubbling of CO_2 through the reaction mixture while filling the test tubes by means of an Adam's aupette. The filled, screw-capped test tubes were shaken vigorously for 2 min, and were incubated at 39 C for 8 hr, during which time they were shaken thoroughly at regular intervals. At the end of the incubation period, the contents of the test tubes were transferred to 50-ml plastic centrifuge tubes, and trichloroacetic acid was added to a final concentration of 10% (Pearson and Smith, 1943). The tubes were then stored for 24 hr in a refrigerator at 1 to 2 C (Bradey, 1960), and the proteins were precipitated by centrifugation at 20,000 $\times$ *g* for 15 min at 0 to 2 C. Deaminating activity was determined by measuring the amounts of free ammonia in the supernatant fluid. Protein biosynthesis was determined by measuring the amounts of protein nitrogen in the precipitate, which had been previously washed twice with a 10% trichloroacetic acid solution.

RESULTS

Effect of carob extract, its tannin and sugar fractions, gallotannic acid, and sucrose on the cel-

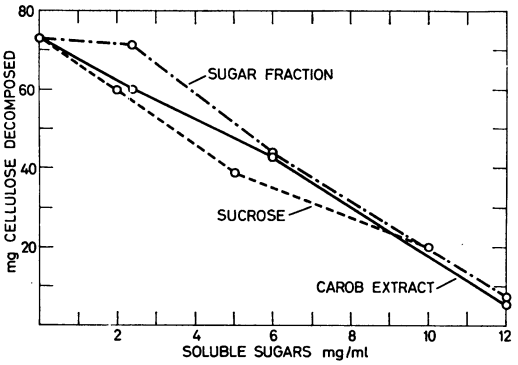

FIG. 2. *Effect of increasing concentrations of carob extract, its sugar fraction, and sucrose on the in vitro cellulolytic activity of rumen fluid.*

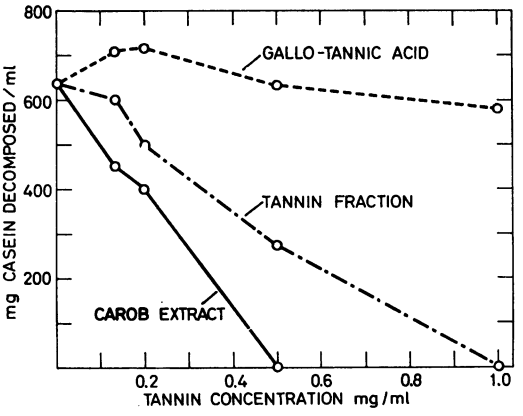

FIG. 3. *Effect of increasing concentrations of carob extract, its tannin fraction, and gallotannic acid on the in vitro proteolytic activity of rumen fluid.*

lulolytic activity of rumen fluid. Cellulose decomposition was almost completely arrested by the addition of carob extract to a final tannin concentration of 1.5 mg/ml (Fig. 1). However, whereas the addition of gallotannic acid resulted in a similar effect, the tannin fraction of the carob extract alone did not inhibit cellulolysis significantly. In Fig. 2, the effect of carob extract on cellulolysis is compared with that of its sugar fraction and that of sucrose. On the basis of sugar content, all three fractions have a similar effect on cellulolysis, with a 12 mg/ml concentration being almost completely inhibitory.

Effect of carob pod extract, its tannin and sugar fractions, gallotannic acid, and sucrose on the proteolytic activity of rumen fluid. In Fig. 3, the effects of carob extract, its tannin fraction, and gallotannic acid on proteolysis are compared on the basis of their tannin content. In contrast to

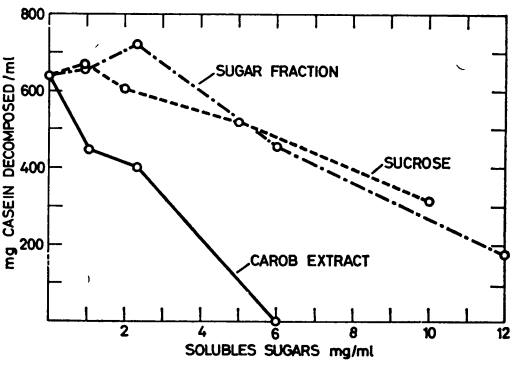

FIG. 4. *Effect of increasing concentrations of carob extract, its sugar fraction, and sucrose on the in vitro proteolytic activity of rumen fluid.*

TABLE 1. *Protein biosynthetic and deaminative (measured as free ammonia) activities of rumen microorganisms as affected by carob extract and its respective sugar and tannin fractions compared with sucrose and gallotannic acid**

Fraction	Final concn (mg/ml)		Net increase (mg/ml)	
	Tannins	Sugars	Protein N synthesis	Free N-NH_3
Control	—	—	0.017	+0.119
Carob extract	0.8	12.7	+0.227	−0.100
Tannin fraction	0.8	—	−0.0003	+0.046
Sugar fraction	—	12.7	+0.288	−0.120
Gallotannic acid	0.8	—	0.038	+0.092
Sucrose	—	12.7	0.304	−0.135

* Initial values for protein N and free N-NH_3 were 0.380 and 0.215 mg/ml of reaction mixture, respectively. Figures are averages of four replicates. LSD values (5%) for protein N biosynthesis and free N-NH_3 are 0.017 and 0.019 mg/ml of reaction mixture, respectively. Standard error of mean: protein biosynthesis, 0.004; free N-NH_3, 0.004. Composition of the reaction mixture: fraction under test, 1.6 ml; rumen liquor plus growth media (13:3), 16.4 ml. Incubation: 8 hr at 39 C.

cellulolysis, proteolysis was very slightly inhibited by gallotannic acid. On the other hand, the tannin fraction of carob extract inhibited proteolysis completely at a concentration of 1 mg/ml. A most significant inhibition of proteolysis by carob extract, on the basis of its tannin or sugar content, was also observed (Fig. 3 and 4); a 100% inhibition was observed at 0.5 mg/ml of tannins and 6 mg of sugars per ml. The significant inhibitory effect of carob extract on proteolysis apparently results from the additive effect of tannins and sugars. It should be noted that the

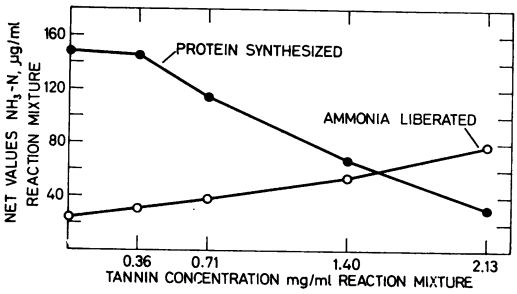

FIG. 5. *Effect of increasing concentrations of tannins obtained from carob extract on the in vitro protein biosynthesis and deaminative activity (measured as free ammonia) of rumen fluid. Composition of the reaction mixture: sugar fraction (22 mg), 1.6 ml; tannin fraction of increasing concentrations, 4.8 ml; rumen fluid plus growth medium (13:3), 12.6 ml. Incubation: 8 hr at 39 C. Free N-NH_3 concentrations at zero time: 121 µg/ml. Protein nitrogen concentrations at zero time: 192 µg/ml. LSD (5%): 9 µg of protein nitrogen per ml of reaction mixture; 8 µg of free-N-NH_3 per ml of reaction mixture.*

tannin-sugar ratio in carob extract is about 20. Therefore, if the reaction mixture to which carob extract had been added contained 0.5 mg of tannins, it also contained 10 mg of soluble sugars per ml. As can be seen from Fig. 4, this sugar concentration inhibited proteolysis by more than 75%. In contrast to the inhibitory effect on proteolysis at relatively high concentrations of sugars, low concentrations enhanced proteolysis. This phenomenon was also noted with gallotannic acid.

Effect of carob extract, its tannin and sugar fractions, gallotannic acid, and sucrose on protein biosynthesis and deaminating activity of rumen fluid. As compared with sugar-containing fractions, tannins alone exert a greater repressive effect on protein biosynthesis by rumen microorganisms (Table 1). This may be explained by the absence of carbohydrates (which are required for biosynthesis) in the tannin fraction. Carob tannins are more detrimental to protein biosynthesis by rumen microflora than is gallotannic acid.

The sugar fraction of carob extract enhanced protein biosynthesis to the same extent as did sucrose. The addition of carob pod extract (tannins plus sugars) increased protein biosynthesis as compared with tannins alone. This favorable effect was partially reduced by the tannins present in the carob pod extract.

Deaminating activity, as measured by the amounts of free ammonia found in the reaction mixture, was significantly reduced by the addition of any of the sugar fractions; this means a greater uptake of NH_3 for protein synthesis.

Effect of increasing concentrations of carob tannins on protein biosynthesis and deaminating activity of rumen fluid. Protein biosynthesis was not affected by carob tannins up to 0.7 mg/ml, but was almost completely blocked at a tannin concentration of 2.1 mg/ml (Fig. 5). On the other hand, the amounts of free ammonia increased significantly with the addition of tannin fraction of carob extract to the reaction mixture. This indicated that deaminating activity was not affected by tannins to the same extent as was protein biosynthesis. The increasing amounts of free ammonia at high tannin concentrations resulted from the inhibition of protein biosynthesis.

DISCUSSION

The effect of carob pod extract on the cellulolytic activity of rumen microorganisms was shown to be correlated with its sugar rather than its tannin content. This is in accordance with experiments in which high sugar content was found to reduce cellulose digestion in the rumen (Krogh, 1954). However, results obtained in previous work (Henis et al., 1964) showed that some saprophytic cellulolytic bacteria were extremely sensitive to the tannin fraction of the pod extract as well as to gallotannic acid. In the present experiments, it was found that proteolytic activity was affected by both tannin and sugar fractions of carob extract, as well as by gallotannic acid and sucrose. Sugars are known to inhibit proteolytic activity of microorganisms (Berman and Rettger, 1918; Kendall and Walker, 1915). Small quantities of sugars, however, encourage protein biosynthesis of rumen microorganisms (Arias et al., 1951; Lewis, 1957; Phillipson, Dobson, and Blackburn, 1959). The apparent inhibition of proteolysis by sugars could have resulted from increased synthesis of bacterial proteins; individual amino acids (Gilroy, 1957; Looper, Stallcup, and Reed, 1959) or breakdown products of protein (Phillipson et al., 1959) could be used as substrate.

Tannins of carob origin were more inhibitive to proteolysis than was gallotannic acid. Inhibition of proteolytic activity by tannins could have resulted from the formation of insoluble complexes with the protein substrate, with proteolytic enzymes, or by blocking the growth and enzyme synthesis of the rumen microorganisms. However, complex formation of tannins with the substrate does not necessarily render it unavailable to proteolytic enzymes (Mejbaum-Katzenellenbogen et al., 1959).

Tannins of various origin have different effects

on the decomposition of organic matter (Basaraba, 1960). This is also the case with gallotannic acid and the tannin fraction of the carob pod extract, which behave quite differently towards cellulolytic and proteolytic activities of rumen microorganisms in the present study.

The inhibitory effect of carob tannins on the biosynthetic activity of the rumen microorganisms was significantly eliminated by the sugar fraction of the carob extract. However, at high tannin concentrations, the harmful effect could not be eliminated.

The amount of free ammonia found in the reaction mixture did not increase correspondingly with the respective inhibition in biosynthetic activity, as would have been expected if ammonia was the only nitrogen source for protein biosynthesis. Easily digestable carbohydrates are known to encourage the utilization of nonprotein nitrogen, such as urea and ammonia (Pearson and Smith, 1943; Mills et al., 1944). It is possible that rumen microorganisms use other nitrogen sources such as amino acids or peptides (Gilroy, 1957; Bryant et al., 1958). On the other hand, the free ammonia level in the reaction mixture might decrease indirectly, either as a result of reduced synthesis of deaminating enzymes or as a result of inhibited protein biosynthesis (Phillipson et al., 1962). Apparently, deamination was less affected by tannins than was biosynthesis.

It may be assumed that microorganisms are not equally affected by tannins of different origin. It is not known which specific groups of microorganisms are the more sensitive, or whether the rumen microflora can be adapted to high tannin content in food by feeding animals on a tannin-rich diet for a long period (Rabi, *personal communication*).

LITERATURE CITED

ALUMOT, E., E. NACHTOMI, AND S. BORNSTEIN. 1964. Low caloric value of carobs as the possible cause of growth depression in chicks. J. Sci. Food Agr. **15**:259–265.

ARIAS, C., W. BURROUGHS, P. GERLANGH, AND R. M. BETHKE. 1951. The influence of different amounts and sources of energy upon *in vitro* urea utilization by rumen micro-organisms. J. Animal Sci. **10**:683–692.

BASARABA, F. 1960. Effects of vegetable tannins on decomposition of some organic compounds. Ph.D. Thesis, Rutgers, The State University, New Brunswick, N.J.

BERMAN, N., AND L. F. RETTGER. 1918. The influence of carbohydrates on the nitrogen metabolism of bacteria. J. Bacteriol. **3**:389–401.

BLACKBURN, T. H., AND P. N. HOBSON. 1960. Proteolysis in the sheep rumen by whole and fractionated rumen contents. J. Gen. Microbiol. **22**: 272–281.

BRADEY, C. J. 1960. Redistribution of nitrogen in grass and leguminous fodder plants during wilting and ensilage. J. Sci. Food Agr. **11**:276–284.

BROWNE, C. A., AND F. W. ZERBAN. 1955. Physical and chemical methods of sugar analysis, 3rd ed., p. 765. John Wiley & Sons, Inc., New York.

BRYANT, M. P., AND L. A. BURKEY. 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci. **36**:205–217.

BRYANT, M. P., N. SMALL, C. BOUMA, AND H. CHU. 1958. *Bacteroides ruminicola* n. sp. and *Succinimonas amylolytica* the new genus and species. J. Bacteriol. **76**:15–23.

CONWAY, E. J. 1957. Microdiffusion analysis and volumetric error, 4th ed., p. 98. Crosby Lockwood & Sons, London.

CRAMPTON, E. W., AND L. A. MAYNARD. 1938. The relations of cellulose and lignin content to the nutritive value of animal feeds. J. Nutr. **15**:383–395.

DRORI, D., AND R. VOLCANI. 1962. The effect of carob pods and sucrose on dairy cattle, as compared with cats. Ktavim **12**:173–178.

DRORI, D., AND R. VOLCANI. 1963. The effect of carobs on the digestibility and N-balance of calves. Ktavim **13**:177–181.

GILROY, J. J. 1957. Thesis, University of Maryland, College Park.

HALLIWELL, G. 1957. Cellulolysis by rumen microorganisms. J. Gen. Microbiol. **17**:153–165.

HENIS, Y., H. TAGARI, AND R. VOLCANI. 1964. Effect of water extracts of carob pods, tannic acid, and their derivatives on the morphology and growth of microorganisms. Appl. Microbiol. **12**:204–209.

KENDALL, A. I., AND A. W. WALKER. 1915. Observations on the proteolytic enzyme of *Bacillus proteus*. J. Infect. Diseases **17**:442–453.

KROGH, N. 1954. Studies on alterations in the rumen fluid of sheep, especially concerning the microbial composition when readily available carbohydrates are added to the food. I. Sucrose. Acta Vet. Scand. **1**:74–83.

LEWIS, D. 1957. Blood-urea concentration in relation to protein utilization in the ruminant. J. Agr. Sci. **48**:438–446.

LOOPER, C. G., O. T. STALLCUP, AND F. E. REED. 1959. Deamination of amino acids *in vivo* by rumen micro-organisms. J. Animal Sci. **18**:954–958.

MEJBAUM-KATZENELLENBOGEN, W., W. DOBRYSZYCKA, J. BOGUSLAWSKA-JAWORSKA, AND B. MORAWIECKA. 1959. Regeneration of protein from insoluble protein-tannin compounds. Nature **184**:1799–1800.

MILLS, R. C., G. G. LARDINOIS, I. W. RUPEL, AND E. B. HART. 1944. Utilization of urea and growth of heifer calves with corn molasses or cane molasses as the only readily available carbohydrate in the ration. J. Dairy Sci. **27**:571–578.

OSLAGE, H. J., AND M. BECKER. 1958. An assay of the nutrient value of carob beans for ruminants,

especially the detrimental effect of the tannic acid content upon protein digestibility. Arch. Tierernaehr. **8**:271–277.

PEARSON, R. M., AND J. A. B. SMITH. 1943. The utilization of urea in the bovine rumen. Biochem. J. **37**:142–164.

PHILLIPSON, A. T., M. J. DOBSON, AND T. H. BLACKBURN. 1959. Assimilation of ammonia nitrogen by rumen bacteria. Nature **183**:402–404.

PHILLIPSON, A. T., M. J. DOBSON, T. H. BLACKBURN, AND M. BROWN. 1962. The assimilation of ammonia nitrogen by bacteria of the rumen of sheep. Brit. J. Nutr. **16**:151–166.

ROSENBLATT, M., AND J. V. PELUSO. 1941. Determination of tannins by photocolorimeter. J. Assoc. Offic. Agr. Chemists **24**:170–181.

SIU, R. G. H. 1951. Microbial decomposition of cellulose, p. 421–425. Reinhold Publishing Co., New York.

TAGARI, H., Y. DROR, I. ASCARELLI, AND A. BONDI. 1964. The influence of levels of protein and starch in rations of sheep on the utilization of protein. Brit. J. Nutr. **18**:333–356.

VOLCANI, R., AND D. DRORI. 1962. Feeding calves with large amounts of carob pods. Ktavim **12**: 167–171.

VOLCANI, R., AND D. LEVI. 1962. The effect of carob pods on the growth of beef calves. Ktavim **12**:95–98.

VOLCANI, R., AND H. RODERIG. 1961. Carob pods as factors in milk production. Ktavim **11**:57–67.

VOLCANI, R., H. TAGARI, AND I. ASCARELLI. 1963. Inhibitory effect of carob pods on cellulose decomposition and deamination in an artificial rumen. Ktavim **13**:51–56.